American Journal of Medical Genetics 63:610–614 (1996)

RAPID PUBLICATION

5,10 METHYLENETETRAHYDROFOLATE REDUCTASE GENETIC POLYMORPHISM AS A RISK FACTOR FOR NEURAL TUBE DEFECTS

C Y Ou, R E Stevenson, V K Brown, C E Schwartz, W P Allen, M J Khoury, R. Rozen, G P Oakley Jr, and M J Adams Jr
Division of Environmental Health Laboratory Sciences (C.Y.O., V.K.B.) and Division of Birth Defects and Developmental Disabilities (M.J.K., G.P.O., M.J.A.), National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia; Greenwood Genetic Center, Greenwood, South Carolina (R.E.S., C.E.B., W.P.A.); Departments of Pediatrics, Human Genetics, and Biology, McGill University-Montreal Children's Hospital Research Institute, Montreal, Canada (R.R.)

Persons with a thermolabile form of the enzyme 5,10 methylenetetrahydrofolate reductase (MTHFR) have reduced enzyme activity and increased plasma homocysteine which can be lowered by supplemental folic acid. Thermolability of the enzyme has recently been shown to be caused by a common mutation (677C→T) in the MTHFR gene. We studied 41 fibroblast cultures from NTD-affected fetuses and compared their genotypes with those of 109 blood specimens from individuals in the general population. 677C→T homozygosity was associated with a 7.2 fold increased risk for NTDs (95% confidence interval: 1.8-30.3; p value: 0.001). These preliminary data suggest that the 677C→T polymorphism of the MTHFR gene is a risk factor for spina bifida and anencephaly that may provide a partial biologic explanation for why folic acid prevents these types of NTD.

KEY WORDS: folic acid, methylenetetrahydrofolate reductase, neural tube defects, spina bifida, anencephaly, encephalocele.

Address reprint requests to Dr Ou, Division of Environmental Health Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Mail Stop F-23, 4770 Buford Highway NE, Atlanta, Georgia 30341-3724.

Received for publication October 26, 1995; revision received April 15, 1996.

INTRODUCTION

Periconceptional supplementation with folic acid is known to reduce the risk of recurrence and occurrence of neural tube defects (NTDs) [U.S. Public Health Service, 1992]. This preventive effect of folic acid has led investigators to search for variants in one or more candidate genes involved in the metabolic pathways of folic acid, defects that could be overcome by periconceptional intake of folic acid. The enzyme MTHFR catalyzes the reduction of 5,10 methylenetetrahydrofolate to 5 methyltetrahydrofolate which is a critical precursor in the cascade of reactions leading to methylation of many biologically important substances and providing, through generation of tetrahydrofolate, essential building blocks for DNA synthesis. Kang et al. [1991] reported that persons with a common thermolabile variant of MTHFR had a 50% reduction in enzymatic activity, increased serum homocysteine, low normal serum folate, and an increased risk for cardiovascular disease. Kang et al.

[1988] also reported that supplemental folic acid can lower plasma homocysteine in persons with this variant.

Recently Goyette et al. [1994] isolated the cDNA for MTHFR, and Frosst et al. [1995] identified a polymorphic variation at the nucleotide position 677 involving a base change from C to T resulting in a substitution of valine (GTC) for alanine (GCC). Persons with 677C→T polymorphisms in the MTHFR gene have increased serum concentrations of homocysteine, and are thought to have an increased risk for cardiovascular disease [Frosst et al., 1995]. Homocysteine abnormalities have been reported in NTD-affected pregnancies [Steegers-Theunissen et al., 1994; 1995; Mills et al., 1995]. Since supplementation with folic acid reduces serum homocysteine in adults with thermolabile MTHFR, it is possible that one of the biologic mechanisms that underlies the NTD-preventive effect of folic acid is related to thermolabile MTHFR. In this report, we present data on the potential role of the 677C→T allele in the cause of NTDs (including spina bifida, anencephaly, and encephalocele). In independent studies, investigators have reported an increased frequency for 677C→T homozygosity for children with spina bifida [van der Put et al., 1995; Whitehead et al., 1995], and for their mothers and fathers [van der Put et al., 1995].

METHODS

The genotypes of 11 anonymous fibroblast cultures from spina bifida-affected fetuses, 21 cultures from anencephaly-affected fetuses, and 9 cultures from encephalocele-affected fetuses (without Meckel syndrome) were determined . These cultures were the most recent consecutive specimens available in the Greenwood Genetic Center cell bank. A sample of 109 healthy persons from the general population collected in Atlanta served as primary controls. Because this was an anonymous control group, age, race, and sex stratification was not possible. A smaller random sample of 54 mothers (38 white, 16 black) who delivered infants in South Carolina in 1995 served as a secondary set of controls.

DNA from cultured fetal fibroblasts was purified by Qiagen DNA affinity chromatography (Qiagen, Chatsworth CA) and approximately 100 ng was subjected to the polymerase chain reaction (PCR) using primers NCO-346 (5'AAGGATGCCCATGTCGGTGCATGCCT3', positions 760 - 733 of the MTHFR cDNA sequence, GenBank accession number U09806) and NCO-347 (5'GAAGCAGGGAGCTTTGAGGCTGACCT, positions 619-644) in a final volume of 50 μl to yield a 142 bp DNA amplified product. One tenth (5 μl) of the amplified product was digested with restriction endonuclease Taq-1 (Promega, Madison, WI) to cleave the DNA sequence that codes for thermolabile MTHFR into two fragments 84 and 58 bp in length. In contrast, a corresponding fragment from a normal MTHFR allele is resistant to Taq-1 digestion and thus, the original 142 bp size fragment remains unchanged. Persons heterozygous for 677C→T will yield all three DNA fragments upon Taq-1 digestion (Fig. 1).

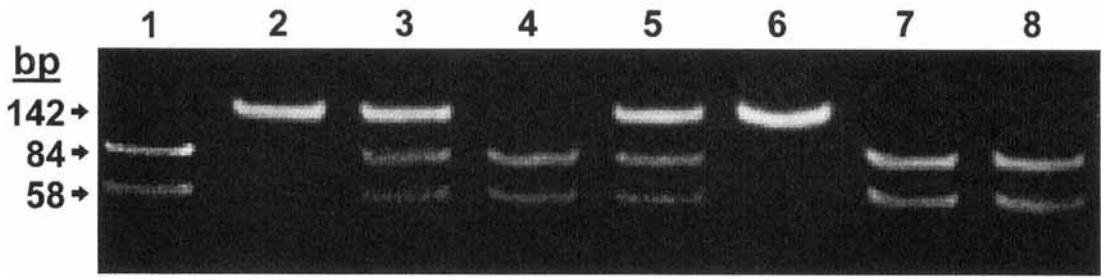

Figure 1: Genotyping of MTHFR 677C→T variants. PCR was carried out with primers NCO-347 and 346 (see text) at 95 °C/30 seconds and 65 °C/45 seconds for 40 cycles in a thermocycler. Amplified DNA was digested with Taq-1 restriction endonuclease and electrophoresed in a 12.5% polyacrylamide gel to reveal the 3 DNA fragments of 142, 84, and 58 base-pairs (bp). Genotypes of DNA specimens in lanes 1, 4, 7, and 8 are homozygous for the thermolabile variant; lanes 3 and 5, heterozygous; lanes 2 and 6 normal.

Statistical analysis of the data was done in 2 by 2 tables using a statistical analysis package (SABER, CDC, 1995). The relative risk for NTDs associated with one or two 677C→T alleles compared with no 677C→T alleles was estimated using odds ratio, 95% confidence intervals and p values were generated using SABER. Finally, the proportion of NTD cases that could be attributed to 677C→T was estimated using the Miettinen formula of attributable fraction (AF):

$$AF = fc\,(OR-1)/(OR)$$

where fc is the fraction of cases with the 677C→T allele and OR is the odds ratio associated with the allele.

RESULTS

As shown in the Table I, 59% of NTD cases had at least one 677C→T allele compared with 38% of the primary set of controls. Thus, the presence of at least one 677C→T allele was associated with a 2.4 fold

TABLE I. Association Between MTHFR Polymorphisms and the Risk of NTD, By number of 677C→T Alleles

Number of 677C→T alleles	NTDs (n=41)	Controls (n=109)	Odds Ratio	95% Confidence Interval	p Value
0 allele					
All	17	68	1.0	Referent	
Spina bifida	3		1.0	Referent	
Anencephaly	8		1.0	Referent	
Encephalocele	6		1.0	Referent	
1 allele					
All	15	36	1.7	0.7 - 4.0	0.30
Spina bifida	4		2.5	0.4 -18.0	0.43
Anencephaly	10		2.4	0.8 - 7.5	0.15
Encephalocele	1		0.3	0.01- 2.8	0.49
2 alleles					
All	9	5	7.2	1.8 - 30.3	0.001
Spina bifida	4		18.1	2.2 -150.0	0.0003
Anencephaly	3		5.1	0.7 - 31.7	0.11
Encephalocele	2		4.5	0.35- 35.5	0.28
1 or 2 alleles					
All	24	41	2.4	1.1 - 5.2	0.03
Spina bifida	8		4.4	1.0 - 27.0	0.05
Anencephaly	13		2.7	0.9 - 8.1	0.07
Encephalocele	3		0.8	0.1 - 4.1	0.92

Four of the cases of anencephaly also had spina bifida; one was homozygous 677C→T, one was heterozygous, and two were homozygous normal.

increased risk for NTDs (95% confidence interval: 1.1-5.2; p value: 0.03). When the analysis was conducted for different subgroups of NTDs, a borderline effect was observed among the spina bifida group (odds ratio of 4.4, 95% C.I. 1.0-27.0, p value 0.05) and the anencephaly group (odds ratio of 2.7, 95% C.I. 0.9-8.1, p value 0.07). There was no effect for the encephalocele group (odds ratio of 0.8, 95% C.I. 0.1-4.1) although the numbers here are very small.

Also shown in the Table I, there was a clear association between 677C→T homozygosity and all NTDs (odds ratio: 7.2; 95% confidence interval: 1.8-30.3; p value: 0.001) and a suggestion of an association with heterozygosity (odds ratio: 1.7; 95% confidence interval: 0.7-4.0; p value 0.30). However, the latter finding was not statistically significant. The calculated attributable fraction of all NTDs is 0.34 if the association with at least one 677C→T allele is causal; the attributable faction is 0.19 if the association with two 677C→T alleles is causal.

The association between 677C→T homozygosity and NTD was seen mainly in whites. All 9 of the NTDs with homozygous 677C→T were among the 34 whites with NTD. None of the 7 blacks with NTD was homozygous. The results reported here reflect comparisons between NTD cases and the larger primary set of controls collected in Southeast US. Genotyping of the smaller sample of South Carolina mothers resulted in even greater differences from those of NTD-affected fetuses. Of the 54 mothers tested, 30 (56%) were homozygous normal, 24 (44%) were heterozygous, and 0 (0%) were homozygous 677C→T. The odds ratio for homozygous 677C→T was infinity (95% C.I, 3.8 - infinity), and for heterozygous 677C→T was 1.1 (95% C.I, 0.4-2.9).

DISCUSSION

The selection of appropriate controls is critical in the study of polymorphisms. In our primary analyses, we used a group of 109 healthy adults from the Southeast as controls for the 41 cases of NTD from South Carolina. A secondary set of controls of 54 mothers who had recently delivered an infant in South Carolina was also available. This set of controls was more geographically similar to the cases but was unique in that the women had carried a pregnancy to term. No 677C→T homozygous polymorphisms were identified in our secondary set of controls.

Our findings suggest that homozygosity for the 677C→T polymorphism of the MTHFR gene in the fetus is a risk factor for NTDs. van der Put et al. [1995] found an increased frequency of the 677C→T homozygous genotype among children with spina bifida and among their mothers in the Netherlands. Whitehead et al. [1995] reported an increased frequency of 677C→T homozygosity among NTD-affected children in an Irish population. These two studies, and ours, that suggest 677C→T homozygosity is a risk factor report relatively low rates of homozygosity among controls (5% to 6%). Since higher rates of homozygosity have been reported [Frosst et al., 1995; de Franchis et al., 1995; Posey et al., 1996], appropriate selection of controls is an important consideration in the analysis of genotype frequencies in different populations. However, more importantly, the genotype alone may not be the critical determinant of outcome but rather the interactive effect between the genotype and folate status, as recently suggested [Jacque et al., 1996]. In that study, the homozygous mutant MTHFR genotype influenced homocysteine levels only when folate levels were below the median value. This genetic-nutrient interactive effect is consistent with the multifactorial model proposed for neural tube defects and suggests a mechanism of how folic acid might overcome a genetic predisposition to this developmental anomaly.

Since 677C→T homozygosity among mothers and children have been reported as risk factors for spina bifida-affected pregnancy, the question remains as to whether the biologic mechanism is mediated through the genotype of the mother, the genotype of the fetus, or some combination of the two. Further study is required to resolve this question.

Our results also suggest that 677C→T polymorphisms may play a more causual role in NTD among whites as compared to blacks. In South Carolina, the NTD prevalence is higher among whites than among blacks (15.9 vs 9.4 per 10,000 live births respectively - unpublished data RES). Further study is needed to

discern whether racial differences in NTD prevalence are related to differences in prevalence of 677C→T homozygosity.

If the association between 677C→T homozygosity and NTD is a causal one, this cause would explain 19% of all NTD's, or about 40% of the 50% of NTDs that are folic acid-preventable. Thermolabile MTHFR as risk factor could explain the homocysteine abnormalities found among affected pregnancies. Additional studies of folate-related mechanisms other than this variant of thermolabile MTHFR (e.g., other mutations of the MTHFR gene or abnormalities in methionine synthase as suggested by Mills et al. [1995]) may also contribute to understanding how folic acid supplementation is effective in those folic acid preventable NTDs not related to 677C→T polymorphisms.

These data do not change the need for implementing public health strategies to provide adequate folic acid consumption among women of childbearing age.

REFERENCES

de Franchis R, Sebastio G, Mandato C, Andria G, Mastroiacovo P (1995): Spina bifida, 677C→T mutation, and the role of folate. Lancet 346;1703.

Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJH, den Heijer M, Kluijtmans LAJ, van den Heuvel LP, Rozen R (1995): A candidate genetic risk factor for vascular disease: A common mutation in methylenetetrahydrofolate reductase. Nature Genet 10:111-113.

Goyette P, Summer JS, Milos R, Duncan AMV, Rosenblatt DS, Matthews RG, Rozen R (1994): Human methylenetetrahydrofolate reductase: Isolation of cDNA, mapping and mutation identification. Nature Genet 7:195-200.

Jacques PF, Bostrom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, Selhub J, Rozen R (1996): Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. Circulation 93:7-9.

Kang SS, Wong PWK, Susmano A, Sora J, Norusis M, Ruggie N (1991): Thermolabile methylenetetrahydrofolate reductase: An inherited risk factor for coronary artery disease. Am J Hum Genet 48:536-545.

Kang SS, Zhou J, Wong PWK, Kowalisyn J, Strokosch G (1988): Intermediate homocysteinemia: A thermolabile variant of methylenetetrahydrofolate reductase. Am J Hum Genet 43:414-421.

Mills JL, McPartin JM, Kirke PN, Lee YL, Conley MR, Weir DG, Scott JM (1995): Homocysteine metabolism in pregnancies complicated by neural-tube defects. Lancet 345:149-151.

Posey DL, Khoury MJ, Mulinare J, Adams MJ Jr, Ou CY (1996): Is mutated methylenetetrahydrofolate reductase (MTHFR) a risk factor for neural tube defects? A pooled analysis. Lancet 347:686-687.

Steegers-Theunissen RPM, Boers GHJ, Blom HJ, Thomas CMG, Nijhuis JG, Borm GF, Eskes TK (1995): Neural tube defects and elevated homocysteine levels in amniotic fluid. Am J Obstet Gynecol 172:1436-1441.

Steegers-Theunissen RPM, Boers GHJ, Trijbels FJM, Finkelstein JD, Blom HJ, Thomas CMG, Borm GF, Wouters GAJ, Eskes TKAB (1994): Maternal hyperhomocysteinemia: A risk factor for neural-tube defects? Metabolism 43:1475-1480.

van der Put N, Steegers-Theunissen R, Frosst P, Trijbels F, Eskes TKAB, van den Heuvel LP, Mariman E, den Heyer M, Rozen R, Blom H (1995): Mutated 5,10 methylenetetrahydrofolate reductase as a risk factor for spina bifida. Lancet 346:1070-71.

Whitehead AS, Gallagher P, Mills JL, Kirke PN, Burke H, Molloy AM, Shields DC, Scott JM (1995): A genetic defect in 5,10 methylenetetrahydrafolate reductase in neural tube defects. Q J Med 88:763-766.

U.S. Public Health Service (1992): Recommendation for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects. MMWR 41:1-7.